

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

3A

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷: C12N 5/06, 15/89, A61K 35/54, 38/24	A1	(11) International Publication Number: WO 00/22098 (43) International Publication Date: 20 April 2000 (20.04.00)
(21) International Application Number: PCT/GB99/03384 (22) International Filing Date: 12 October 1999 (12.10.99) (30) Priority Data: 9822235.9 12 October 1998 (12.10.98) GB 60/104,116 13 October 1998 (13.10.98) US (71) Applicant (for all designated States except US): GERON BIO-MED LIMITED [GB/GB]; Roslin Biotechnology Centre, Roslin, Midlothian EH25 9PS (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): DE SOUSA, Paul, Alexandre [CA/GB]; Geron Bio-Med Limited, Roslin Biotechnology Centre, Roslin, Midlothian EH25 9PS (GB). KING, Timothy, James [GB/GB]; Geron Bio-Med Limited, Roslin Biotechnology Centre, Roslin, Midlothian EH25 9PS (GB). WILMUT, Ian [GB/GB]; Geron Bio-Med Limited, Roslin Biotechnology Centre, Roslin, Midlothian EH25 9PS (GB). ZHU, Jie [CN/GB]; Geron Bio-Med Limited, Roslin Biotechnology Centre, Roslin, Midlothian EH25 9PS (GB). (74) Agent: BASSIL, Nicholas, Charles; Kilburn & Strode, 20 Red Lion Street, London EC1R 4PJ (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PORCINE OOCYTES WITH IMPROVED DEVELOPMENTAL COMPETENCE (57) Abstract A method is described of selecting a porcine oocyte or a population of oocytes with improved developmental competence following either <i>in vitro</i> or <i>in vivo</i> maturation.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PORCINE OOCYTES WITH IMPROVED DEVELOPMENTAL COMPETENCE

FIELD OF THE INVENTION

5 This invention relates to the harvesting of porcine oocytes with improved developmental competence and to the generation of pigs by assisted reproduction strategies, including nuclear transfer including but not being limited to, genetically selected and/or modified animals.

BACKGROUND OF THE INVENTION

10 The female reproductive cycle begins with maturation and ovulation of the oocyte within the female reproductive tract. In normal sexual reproduction, fertilization of the oocyte by sperm also occurs in the reproductive tract. Assisted reproduction strategies, such as *in vitro* fertilization (IVF) and nuclear transfer, use oocytes that are harvested from the female animal at some point prior to fertilization and
15 subsequently processed *in vitro*. Since the fertile life span of an oocyte is short, defining the optimal period within which to retrieve oocytes for reproductive strategies is invaluable, as is insight into how that period can be manipulated. Currently, assisted reproduction strategies use oocytes that are selected at random from a matured oocyte population. Typically, such oocyte populations are removed
20 from the ovaries of slaughterhouse animals and matured *in vitro*, or are harvested from superovulated pigs and are thus matured *in vivo*.

There is little known of the factors that define the competence of an animal oocyte to develop following ovulation and/or maturation. Although developmental competence
25 is positively correlated with the size of the follicles from which they are derived (Pavlok *et al.*, *Mol. Reprod. Dev.* **35** 233-243 (1993)), not all large follicles ovulate and in fact most undergo atresia, with concomitant loss of the oocyte within (Driancourt, *Theriogen* **35** 55-79 (1991)). A more likely factor is the requirement for a specific follicular steroid environment (Moor *et al.*, *JEEM* **56** 319-335 (1980)).
30 In support of this studies in cattle and sheep have demonstrated follicular differences

in growth and steroid hormone expression correlated with dominant and secondary waves of folliculogenesis (reviewed by Roche *Rev of Reprod* 1 19-27 (1996)).

To overcome the hurdles imposed by the finite life and undefined characteristics of a developmentally competent oocyte two approaches have been taken. The first approach uses exogenous gonadotropins to induce (super) ovulation, and the second bypasses ovulation and recapitulates the maturation of oocytes *in vitro* under defined conditions (reviewed in Boland and Roche, *Mol. Reprod. Dev.* 36 266-270 (1993)). Both methods improve the success of reproductive strategies by providing increased numbers of oocytes for subsequent insemination and/or manipulation. However, these methods do not guarantee optimal developmental competence of the oocytes that they provide. Superovulation of prepubertal sheep, pigs, and cattle still results in oocytes of diminished developmental competence compared with oocytes derived from post-pubertal animals (Wright *et al.*, *J. Anim. Sci.* 42 912-917 (1976); Pinkert *et al.*, *J Reprod. Fert.* 87 63-66 (1989); Seidel *et al.*, *Dairy Sci.* 54 923-926 (1971)). In addition, oocytes matured *in vitro* as cumulus-oocyte-complexes are highly heterogeneous in their morphology and quality. The abundance of cumulus coverage surrounding oocytes has been positively correlated with their developmental competence following *in vitro* maturation (Leibried-Rutledge and First, *J. Anim. Sci.* 48 76-86 (1979); De Loos *et al.*, *Gamete Res.* 24 197-204 (1989); Blondin and Sirard, *Mol. Reprod. Dev.* 41 54-62 (1995); Funahashi and Day, *J. Reprod. Fert. Suppl.* 52 271-283 (1997)).

Previous studies in the pig have monitored ovulation by either slaughter, laparoscopy, or transcutaneous or transrectal ultrasound (Hunter *Res. Vet. Sci.* 13 356-361 (1972); Brussow *et al.*, *Reprod. in Dom. Anim.* 25 255-260 (1990); Weitze *et al.*, *Reprod. Dom. Anim.* 25 61-67 (1990); Weitze *et al* *Zuchthyg.* 24 40-42 (1989); Soede *et al.*, *Theriogen* 38 653-666 (1992)). In the pig the timing of ovulation during the oestrus phase of the reproductive cycle is highly variable. For example, in spontaneously ovulating animals, timing may vary from between 10 to 85 hours after the onset of oestrus (reviewed by Soede and Kemp *J. Reprod. Fert.*

Suppl. 52 91-103 (1997)). This can be shortened by 5 to 14 hours following intracervical infusion of seminal plasma (Weitze *et al.*, *Reprod. Dom. Anim.* 25 61-67 (1990)). Such infusion has no effect on the synchronized ovulatory response of gonadotropin treated gilts (Brussow *et al.*, *Reprod. Dom. Anim.* 28 119-122 (1992)),
5 which ovulate between 35 to 50 hours post stimulation depending on the gonadotropin used (reviewed by Kemp and Soede, *J. Reprod. Fertil. Suppl.* 52 79-89 (1997)).

Seasonally reduced fertility in pigs, known as "summer infertility", has been
10 reported, although this effect is less pronounced than in wild pigs or other species such as the sheep (reviewed by Claus and Weiler *J. Reprod. Fert. Suppl.* 33 185-197 (1985)). In sheep, variations in the duration of day length have been demonstrated as the regulatory stimulus controlling the pattern of seasonal reproduction. Effects of photoperiod are transduced from neuronal to endocrine signals by the stimulation of
15 the pineal gland, which produces hormones such as melatonin that in turn stimulate gonadotropin release. Diurnal variations in porcine plasma melatonin have been described (Klupiec *et al.*, *J. Pineal Res.* 22 65-74 (1997)). However, there is no evidence that melatonin or its precursors and metabolites are involved in regulating ovulation or reproductive performance (Foxcroft *et al.*, in "Principles of pig
20 science", ed. DJA Cole, Nottingham University Press (1994)).

In general the mechanisms establishing ovulation rate in the pig are poorly understood. In the pig, growth and development of a follicle is dependent on pituitary gonadotropins from the time it acquires theca interna cells. The growth of
25 selected preovulatory follicles in the pig is also associated with rapid atresia of smaller follicles (Foxcroft and Hunter, *J. Reprod. Fertil. Suppl.* 33 1-19 (1985)). This is consistent with the existence of a dominant follicular wave suggested for monotocous species such as cattle. Recruitment of follicles from the antral follicle pool (and thus rescue from atresia) is also known to be induced by a specific pattern
30 of episodic Lutenizing hormone release (reviewed by Kemp *et al.*, "Control of

Ovulation" in "Progress in Pig Science", ed. Wisemean, Nottingham University Press (1988)).

5 A limited number of studies have attempted to examine the relationship between embryonic survivability and either the duration of ovulation or its early induction in the pig. Initial reports suggested that the first ovulating follicles resulted in the best developing embryos in a litter (Pope *et al.*, *Biol. of Reprod.* 39 822-887 (1988); Xie *et al.*, *Biol. of Reprod.* 43 236-240 (1990)). However, these results have been challenged by others (Soede *et al.*, *Theriogen* 38 653-666 (1992); Soede and Kemp 10 *Theriogen* 39 1043-1053 (1993)). Early embryonic survivability has also been found to be unaffected by early induction of ovulation, induced by varying the timing of gonadotropin injections relative to each other (reviewed by Kemp *et al.*, "Control of Ovulation" in "Progress in Pig Science", ed. Wisemean, Nottingham University Press (1988)). There have been no reports suggesting improved embryonic 15 survivability following delayed ovulation of the oocyte used to prepare the embryo. A single study has described the collection of porcine oocytes at later time points relative to injection of gonadotropin in which the oocytes are said to show an improved capacity to be activated and develop to the blastocyst stage following nuclear transfer. However, in this study the timing of ovulation was not recorded 20 and nor was the stage in the diurnal cycle of the animal noted. The beneficial effect was attributed by the authors to ageing of the oocytes within the oviduct (Nagashima *et al.*, *Mol. Reprod. Dev.* 48 339-343 (1997)).

25 It is known that ovulation in pigs occurs over a broad time range (observed to be 10-85 hours post-oestrus) under normal conditions where oocytes ovulate spontaneously. More controlled ovulation has been obtained using superovulation protocols where ovulation is generally seen 35-50 hours post-injection with a gonadotropin hormone to induce ovulation. However, it is difficult to obtain oocytes of sufficiently high quality from either route for use in assisted reproduction, 30 including nuclear transfer, even with the introduction of superovulation protocols. In assisted reproduction strategies such as *in vitro* fertilization (IVF) and nuclear

transfer there is therefore a need to improve the efficiency of the process by selecting the best possible oocytes from a matured population of oocytes.

SUMMARY OF THE INVENTION

5 An optimum time frame or "window" of developmental competence for porcine oocytes as measured by parthenogenetic activation of the oocytes has now been discovered. The optimum time frame or window has been demonstrated in oocytes obtained by both *in vivo* and *in vitro* maturation. The invention provides methods for selecting oocytes having maximal developmental competence which can be
10 suitably measured by parthenogenetic activation of oocytes prepared according to a method of the invention. Parthenogenetic activation is a convenient method by which the developmental competence of oocytes can be measured. It is generally considered to accurately reflect the competence of oocytes to develop when used in nuclear transfer or in IVF protocols.

15 Outside of this window of developmental competence now discovered, the ability of porcine oocytes to undergo parthenogenetic activation declines markedly, i.e. the oocytes are less capable of development in assisted reproduction strategies. In unselected populations of oocytes there will be a large proportion of cells that are
20 outside of the window.

According to a first aspect of the invention there is provided a method of producing a porcine oocyte optimised for developmental competence, the method comprising culturing a harvested oocyte *in vitro* for between about 36 to about 48 hours.
25 Methods in accordance with this aspect of the present invention also extend to selection of a population of porcine oocytes with improved developmental competence.

According to a second aspect of the invention there is provided a method of
30 producing a porcine oocyte optimised for developmental competence, the method comprising harvesting an oocyte from a pig from about 42 to about 50 hours post-

injection with a gonadotropin hormone, wherein the oocyte was ovulated from about 42 to 50 hours post-gonadotropin injection. Methods in accordance with this aspect of the present invention also extend to selection of a population of porcine oocytes with improved developmental competence. Detection of ovulation in the pig can
5 conveniently be monitored using transcutaneous ultrasonography

Methods in accordance with the second aspect of the invention can conveniently be carried out by delaying the time of ovulation in the donor female animal or by manipulating the fertility cycle of the pig by administering progesterone or an
10 analogue thereof in the middle of the cycle (mid-luteal administration) to the animal. Where mid-luteal administration of progesterone or an analogue thereof is to be employed, the animal to be treated is first monitored to establish the course of its estrous cycle. Once an actively cycling animal is identified, the animal is fed progesterone or an analogue thereof for 4-5 days (at around day 11-15 of the cycle)
15 to maintain anestrous (i.e. mid-luteal administration). Once the hormone supplement is removed, the animal reinitiates another cycle. Injection with a gonadotropin hormone 1 and 4 days later results in ovulation in a controlled manner. This represents an improvement over traditional superovulation protocols which involve progesterone treatment for a full 18 days of the estrous cycle.

20 A previously unreported and alternative method of delaying ovulation is also encompassed by the invention. As part of experiments aimed at optimizing the timing of the superovulatory response of mature gilts to exogenous gonadotropins, it has now been discovered that the timing of ovulation can be delayed by
25 approximately 3 hours relative to the time of the last gonadotropin injection, without affecting the yield of oocytes. This can be achieved by administering the gonadotropin to the animal by any convenient route, for example by injection, i.e. intravenous, intramuscular, intraperitoneal, subcutaneous, or by other means such as oral, topical, etc. Using transcutaneous ultrasonography to precisely monitor
30 ovulation, oocytes can be retrieved from oviducts following ovulation, and activated to initiate their early developmental program. This alternative method according to

the present invention has been found to provide oocytes which have an improved developmental competence. The shift in the time of administration of the gonadotropin hormone in the diurnal rhythm of the animal has been shown to shift the time of ovulation such that in the *in vivo* maturation period is increased to bring
5 the time of ovulation within the time period or window of optimal developmental competence. The developmental competence of such oocytes to form the end stages of preimplantation development is positively correlated with increased time to ovulation.

10 Preferred methods in accordance with either the first or the second aspects of the invention are as follows. In an *in vitro* maturation method of producing a porcine oocyte optimised for developmental competence, the method may comprise: (i) harvesting a cumulus oocyte complex (COC) from a follicle of a pig ovary; and (ii) maturing said COC in a physiologically acceptable culture medium comprising a
15 gonadotropin hormone for between about 36 to about 48 hours. In embodiments where even greater enhanced developmental competence is required, the oocyte or COC may be matured in *in vitro* for about 42 to about 46 hours or about 43 hours to about 45 hours.

20 In an *in vivo* method of producing a porcine oocyte optimised for developmental competence, the method may comprise: (i) injecting an animal with a gonadotropin hormone; and (ii) harvesting an oocyte from a pig from about 42 to about 50 hours post-injection with gonadotropin, wherein the oocyte was ovulated from about 42 hours to about 50 hours as determined by transcutaneous ultrasonography. In
25 embodiments where even greater enhanced developmental competence is required, the oocyte may be harvested from about 44 hours to about 48 hours, wherein the oocyte was ovulated from about 44 hours to about 48 hours as determined by transcutaneous ultrasonography, or alternatively, the oocyte may be harvested from about 45 hours to about 47 hours, wherein the oocyte was ovulated from about 45
30 hours to about 47 hours as determined by transcutaneous ultrasonography.

Ovulation may be seen to occur within about 1 hour to about 6 hours following gonadotropin injection.

Further more specific *in vivo* methods of producing a porcine oocyte optimised for developmental competence may be as follows. Firstly, the method may comprise: (i) administering progesterone or an analogue thereof to a pig for 18 days to establish a reference heat in the animal; (ii) thirteen to sixteen days subsequently administering to the animal a first injection of Pregnant Mares Serum Gonadotropin (PMSG); (iii) after a time period of 88 hours administering to the animal a second injection of human chorionic gonadotropin (hCG); and (iv) harvesting an oocyte from a pig from about 45 to about 47 hours post-injection with hCG, wherein the oocyte was ovulated from about 45 to 47 hours post-hCG injection as determined by transcutaneous ultrasonography. As an alternative to a method as described above where the hCG injection can be optimally shifted in the diurnal rhythm of the animal such that it takes place 88 hours after the PMSG injection, a preferred method can comprise: (i) administering to the animal progesterone or an analogue thereof for four or five days at days 11 to 15 of the estrous cycle; (ii) subsequently administering to the animal PMSG one day later and hCG four days later to cause ovulation of an oocyte; and (iii) harvesting an oocyte from a pig from about 45 to about 47 hours post-injection with hCG, wherein the oocyte was ovulated from about 45 to 47 hours post-hCG injection as determined by transcutaneous ultrasonography.

Methods in accordance with these aspects of the present invention therefore permit the selection of pig oocytes with an optimised developmental competence in a defined time period. Such oocytes have an increased ability to undergo development following activation. The methods overcome the problems encountered with the previous protocols for porcine oocytes in nuclear transfer and assisted reproduction.

According to a third aspect of the present invention there is provided a method of harvesting an oocyte from a porcine mammal in response to the administration of a gonadotropin hormone, the method comprising administration of the gonadotropin

hormone towards the end of the diurnal rhythm of the porcine mammal, and subsequently harvesting the ovulated oocyte.

5 This aspect of the present invention provides a process by which ovulation in porcine mammals, can be delayed to prolong the residence time of an oocyte within a follicle but where ovulation still occurs at a predictable time so that viable, developmentally competent oocytes may be retrieved within a defined window of time. Methods in accordance with this aspect of the present invention can also be used to enhance the precision of existing protocols to manipulate the timing of ovulation and the quality
10 of the oocytes that are subsequently obtained for natural and assisted reproductive strategies. A major advantage of this aspect of the invention is that it permits the efficacy of these strategies to be increased by increasing the precision with which oocytes are ovulated and handled and improving the likelihood that the oocytes retrieved have a high developmental capacity.

15 This aspect of the present invention also extends to a method of enhancing the developmental competence of a harvested porcine oocyte, the method comprising administration of a gonadotropin hormone to a porcine mammal towards the end of the diurnal rhythm of the animal prior to harvesting the oocyte such that ovulation is
20 delayed.

According to a fourth aspect of the invention there is provided a method of nuclear transfer, the method comprising the introduction of a nucleus from a donor cell into a porcine oocyte produced in accordance with the first or second aspects of the
25 present invention described above.

According to a fifth aspect of the invention there is provided a method of *in vitro* production of a porcine embryo, the method comprising the step of *in vitro* fertilization (IVF) of an oocyte prepared in accordance with the preceding aspects of the
30 invention

The present invention also extends to an oocyte prepared according to a method as described above. According to a sixth aspect of the invention there is provided a composition of porcine oocytes selected for optimal developmental competence, wherein the oocytes substantially comprise oocytes matured *in vitro* for between
5 about 36 to about 48 hours. In embodiments of the invention where even greater enhanced developmental competence is desired, the oocytes may be matured for between about 42 to about 46 hours or alternatively for between about 43 to about 45 hours.

10 According to a seventh aspect of the invention there is provided a composition of porcine oocytes selected for optimal developmental competence, wherein the oocytes substantially comprise oocytes harvested from at least one pig from about 42 to about 50 hours post-injection with a gonadotropin hormone, wherein the oocyte was ovulated from about 42 to about 50 hours post-gonadotropin injection. In
15 embodiments of the invention where even greater enhanced developmental competence is desired, the oocytes may be harvested from the pig from about 44 to about 48 hours post-injection with a gonadotropin hormone, wherein the oocytes were ovulated from about 44 to about 48 hours post-gonadotropin injection. The oocytes can also be harvested from the pig from about 45 to about 47 hours post-
20 injection with a gonadotropin hormone, wherein the oocytes were ovulated from about 45 to about 47 hours post-gonadotropin injection. As in previous aspects of the invention, ovulation of the oocytes may be conveniently detected using transcutaneous ultrasonography.

25 Also included is the use of separate first and second administrations of gonadotropin hormone to harvest an oocyte from a porcine mammal in which the first administration of gonadotropin hormone is towards the end of the diurnal rhythm of the animal. The gonadotropin hormone may also be used in the manufacture of an agent for the harvesting of an oocyte from a porcine mammal whereby the
30 gonadotropin hormone is administered towards the end of the diurnal rhythm of the animal. This aspect of the invention also extends a kit comprising a gonadotropin

hormone wherein the kit contains instructions for administration of the gonadotropin hormone to a porcine mammal for harvesting an oocyte from the animal in which administration of the hormone is towards the end of the diurnal rhythm of the animal. Alternatively, the kit may comprise a first gonadotropin hormone and a second gonadotropin hormone wherein the kit contains instructions for administration of the gonadotropin hormones to a porcine mammal for harvesting an oocyte from the animal in which administration of the first hormone is towards the end of the diurnal rhythm of the animal and administration of the second hormone is separated from the first administration by about three days.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

Methods of the present invention are applicable to mammals of the species *porcus*, including pig species such as Large White, Landrace, Meishan, Minipig. The term "porcine" as used herein refers to any pig species.

The term "oocyte" is used to describe the mature animal ovum which is the final product of oogenesis and also the precursor forms being the oogonium, the primary oocyte and the secondary oocyte respectively. Methods in accordance with the invention may also find application to oocytes collected from transgenic or genetically modified porcine mammals, including chimeras and also oocytes obtained from animals derived from embryos prepared by nuclear transfer procedures. The collection of oocytes from animals for *in vitro* maturation involves the aspiration of cumulus oocyte complexes (COCs) from the follicles of animals. References to harvesting of oocytes from the follicles of animals prior to *in vitro* maturation of the oocytes should therefore be understood to include cumulus oocyte complexes.

It should be noted that the term "transgenic", in relation to animals, should not be taken to be limited to referring to animals containing in their genome or germ line one or more genes from another species, although many transgenic animals will contain such a gene or genes. Rather, the term refers more broadly to any animal

whose germ line or genome has been the subject of technical intervention by recombinant DNA technology. So, for example, an animal in whose germ line an endogenous gene has been deleted, duplicated, activated or modified is a transgenic animal for the purposes of this invention as much as an animal to whose genome or
5 germ line an exogenous DNA sequence has been added.

In embodiments of the invention in which the animal from which the oocyte is collected is transgenic, the genetic modification to the animal may be undertaken using physical techniques such as microinjection into the male or female pronucleus
10 of the zygote or into the cytoplasm or nucleus of an oocyte or embryo from which the animal develops. Alternatively, the genetic modification can involve the use of mass transformation or transfection techniques such as electroporation, viral transfection (including the use of adenoviruses, retroviruses, adeno-associated means or synthetic retrotransposons), lipofection, microprojectile cell bombardment,
15 antisense technology, vectors such as YAC and BAC or by using other means such as sperm. Furthermore the modification can benefit from intervention by homologous recombination, DNA repair mechanisms, including the use of restriction enzymes. Cell-mediated transgenesis can employ a variety of cells, including ES cells, EG cells and other stem cells or suitable cells from any mammalian species.

20
Developmental competence is herein defined as the ability of an oocyte to undergo parthenogenetic activation and develop at least to the morula or to the blastocyst stage in terms of embryo development. As discussed above, parthenogenetic activation is seen as a reliable indicator of the ability of an oocyte to develop under
25 normal circumstances following fertilization, such as for example IVF, or following nuclear transfer.

In superovulation protocols, the first step often comprises the administration of progesterone or an analogue thereof to the animal to enable establishment of a
30 reference heat. The progesterone analogue may be, but is not limited to, altrenogest or regumate.

Gonadotropin hormones (gonadotrophins or gonadotropic hormones) are a group of vertebrate glycoprotein hormones, controlling production of specific hormones by gonadal endocrine tissues. Anterior pituitaries of both sexes produce follicle-stimulating hormone (FSH) and lutenizing hormone (LH, or interstitial cell stimulating hormone (ICSH) in males); but their effects in the two sexes are different. Human chorionic gonadotropin (hCG) is an embryonic product whose presence in maternal urine is usually diagnostic of pregnancy. Release of FSH and LH is controlled by hypothalamic gonadotropin-releasing factors (GnRFs), including for example Gonadotropin-releasing hormone (GnRH). Many gonadotropin preparations are known in the art. In methods according to the present invention, the gonadotropin preparation may be, for example, Pregnant Mares Serum Gonadotropin (PMSG), human chorionic gonadotropin (hCG), equine Chorionic Gonadotropin (eCG) or Gonadotropin Releasing Hormone (GnRH). A prostaglandin or synthetic analogue thereof may also be co-administered with the gonadotropin as appropriate. An example of a synthetic prostaglandin hormone is cloprostinol. Other examples are well known in the art.

The administration of the gonadotropin hormones may be undertaken by any generally suitable procedure, including intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, or by other routes including oral, topical, vaginal, or rectal. Solid dosage or particulate dosage forms may be administered as controlled release formulations where appropriate. Preferably the administration is via an injection.

Ovulation in pigs that have been subjected to a superovulation protocol occurs after injection of the animal with a gonadotropin hormone.

The diurnal rhythm or circadian rhythm of an animal can be defined as endogenous (intrinsic) rhythmic changes occurring in an organism with a periodicity of approximately 24 hours. The rhythm will even persist for some days in the

experimental absence of the daily rhythm of environmental cycles (e.g. light/dark) to which diurnal rhythm is usually entrained. In animals, diurnal rhythm is seen as sleep rhythms and running activity. Rhythms of hormone secretion have been implicated in some circadian rhythms but their existence implies some form of biological clock whose detailed biochemistry remains unknown. In methods according to the present invention, the administration of exogenous gonadotropins is towards the end of the animal's daily diurnal rhythm, i.e. it may be in the second half of the diurnal rhythm, preferably in the third quarter of the diurnal rhythm or in the fourth quarter of the diurnal rhythm. Assuming a general periodicity in the animal's diurnal rhythm of 24 hours, these periods are respectively, 12.00am to 12.00pm, 12.00am to 6.00pm and 6.00pm to 12.00pm local standard time.

Local standard time is defined as being the official time zone in the international time zone system at the place where the method is carried out. References in the present application to specific times should be interpreted as referring to the local standard time applying at the place where the method is carried out. Appropriate adjustments will of course need to be made to such times where different time systems are used, such as for example, during the winter months as opposed to the summer months.

In embodiments of the invention relating to compositions of oocytes, the oocytes substantially comprise oocytes prepared according to a defined method of the invention. Typically, such an oocyte population will comprise at least about 25% oocytes prepared according to a defined method of the invention. However, it will be understood that the overall developmental competence of the oocytes will increase as the proportion of the oocytes prepared according to a defined method of the invention increases. Thus, in a particular embodiment, an oocyte composition may comprise at least about 50%, at least about 75%, at least about 90%, or at least about 95% of oocytes prepared according to a defined method of the invention may be utilised. The term "substantially comprises" with reference to such oocyte compositions encompasses such preparations.

2. In vitro maturation

Porcine oocytes can be harvested by any suitable means in the art for subsequent *in vitro* maturation. For example, oocytes can be obtained by surgical procedures, including laparotomy, carried out on animals under anaesthesia. The procedures can
5 be carried out on live animals, although it is possible to collect oocytes from the ovaries of recently slaughtered animals *post mortem*. The selection of oocytes from the ovaries is carried out manually from follicles which are about from 3-8mm in size are selected. In practice, a cumulus oocyte complex (COC) is aspirated from a follicle and the COC is subsequently matured *in vitro*. The matured oocytes are
10 denuded of cumulus cells prior to use, i.e. the cumulus cells are removed. References to oocytes include COCs, where appropriate, and also *vice versa*.

The improved developmental competence of oocytes selected by a method in accordance with this aspect of the invention, is provided by culturing a harvested
15 oocyte *in vitro* for between about 36 to about 48 hours. The time period is measured with reference to the time of transfer of isolated cumulus oocyte complexes into culture medium containing gonadotropin hormone(s). The culture period may be from about 36 to about 48 hours, suitably from about 42 to 46 hours and preferably from about 43 to about 45 hours. The culture may be carried out in any suitable *in vitro* maturation medium, e.g. according to Wang *et al* (*J. Reprod. Fertil.* 111 101-
20 108 (1997)). Selection of an oocyte matured under these conditions (or a population of oocytes) provides an oocyte which has an improved competence for development compared to a control oocyte.

3. In vivo maturation

The porcine oocyte may be harvested by any suitable means in the art. For example, oocytes can be obtained by surgical procedures, including laparotomy, carried out on animals under anaesthesia. Alternative procedures include the use of media to flush
out oocytes from the fallopian tubes. Suitably such procedures are carried out on
30 live animals.

The improved developmental competence of oocytes selected by a method in accordance with this aspect of the invention, is provided by harvesting an oocyte from a pig from at about 42 to about 50 hours post injection with a gonadotropin hormone, wherein the oocyte is ovulated from about 42 to 50 hours post-gonadotropin injection. The time period can be measured with reference to the time of injection of the animal with a gonadotropin hormone to induce superovulation where transcutaneous ultrasonography is used to confirm that ovulation has occurred. Suitable gonadotropin hormone preparations are known in the art and include, but are not limited to, Pregnant Mares Serum Gonadotropin (PMSG), human chorionic gonadotropin (hCG), equine Chorionic Gonadotropin (eCG) or Gonadotropin Releasing Hormone (GnRH). While oocytes harvested within the 42-50 hour post-gonadotropin period show enhanced developmental competence, greater developmental competence may be obtained by harvesting oocytes at from about 44 to 48 hours or from about 45 to about 47 hours. Selection of an oocyte ovulated under these conditions (or a population of oocytes) provides an oocyte which has an improved competence for development.

The present application will find great utility in controlled super-ovulation in animals. Generally, this procedure involves at least one injection of a gonadotropin hormone to the animal from which oocytes are to be collected. Preferably, the procedure involves the administration of a first injection and a second injection which are separated by about three to four days, for example three and a half days. The two injections can be separated by about 72 to 96 hours, suitably about 78 to 92 hours, more preferably separated by about 83 to 88 hours, in which the first injection is administered towards the end of the diurnal rhythm of the animal as described above.

Using local standard time as a base from which to calculate the times for administration of the injections, it can be seen that the first injection should preferably be made during the second half of the diurnal rhythm of the animal, i.e. in the afternoon or early evening. Generally, the first injection can be given between

6.00pm to 8.00pm local standard time, preferably at 7.00pm local standard time. The second injection can then be given subsequently allowing for the separation as described above. The second injection can therefore be given at 10.00am to 12.00am local standard time, preferably at 11.00am local standard time, where the separation is 88 hours between the first and second injection. However, the skilled person will be able to determine other injection time protocols with reference to the parameters already outlined.

In a preferred embodiment of the invention, the gonadotropin hormone administered in the first injection can be Pregnant Mares Serum Gonadotropin (PMSG). In a further preferred embodiment of the invention, the gonadotropin hormone administered in the second injection can be human chorionic gonadotropin (hCG) or Gonadotropin Releasing Hormone (GnRH). The gonadotropin may administered in combination with other hormones, for example steroid hormones, such as a prostaglandin, including synthetic prostaglandins e.g. cloprostinol.

The injection of PMSG is generally understood to be responsible for recruitment of follicles which contain the oocytes to be ovulated. The second injection of a gonadotropin hormone such as hCG, induces ovulation and maturation of the oocyte.

Ovulation in an animal can be defined to have occurred when the majority of follicles surrounding an oocyte have disappeared as seen using transcutaneous ultrasonography. Methods practised in accordance with the present invention can therefore involve, but are not limited to, the use of transcutaneous ultrasonography to evaluate precisely and non-invasively the timing of ovulation. Such monitoring of the time of ovulation permits selection of oocytes for collection which have undergone a delayed ovulation as a result of the superovulation protocol used as described above. Monitoring the time of ovulation is valuable as it enables the determination of the developmental competence of an oocyte by selection according to a method of this aspect of the present invention. In order to achieve the full

advantages of this aspect of the present invention, the time of ovulation should be known.

4. Applications of the Invention

5 Currently, mature oocytes are utilised in a number of technical procedures, including nuclear transfer and methods of *in vitro* production (IVP) of animals, such as *in vitro* fertilization (IVF). The oocytes and oocyte populations having optimal developmental competence prepared by the methods of the present invention may be used in these and other procedures and applications.

10

(a) Application of invention in methods of nuclear transfer

In the method of this aspect of the invention described above, a nucleus is transferred from a donor cell to a recipient porcine oocyte prepared and matured as described herein. The use of this method is not restricted to a particular donor cell type. The donor cell may be as described in Wilmut *et al Nature* 385 810 (1997); Campbell *et al Nature* 380 64-66 (1996); or Cibelli *et al Science* 280 1256-1258 (1998). All cells of normal karyotype, including embryonic, foetal and adult somatic cells which can be used successfully in nuclear transfer may in principle be employed in a method according to the present invention. Foetal fibroblasts are a particularly useful class of donor cells. Generally suitable methods of nuclear transfer are described in Campbell *et al Theriogenology* 43 181 (1995), Collas *et al Mol. Reprod. Dev.* 38 264-267 (1994), Keefer *et al Biol. Reprod.* 50 935-939 (1994), Sims *et al Proc. Nat'l. Acad. Sci. USA* 90 6143-6147 (1993), WO-A-9426884, WO-A-9424274, WO-A-9807841, WO-A-9003432, US-A-4994384 and US-A-5057420. The invention therefore contemplates the use of an at least partially differentiated cell, including a fully differentiated cell. Donor cells may be, but do not have to be, in culture and may be quiescent. Nuclear donor cells which are quiescent are cells which can be induced to enter quiescence or exist in a quiescent state *in vivo*.

30 The recipient cell into which the donor nucleus has been transferred may be cultured *in vitro* or *in vivo* until a suitable stage in embryonic development is reached. The

invention includes the derivation of a cell line from desired cells of the embryo, e.g. inner cell mass cells, for example in the derivation of a stem cell line. Suitably, the embryo may be cultured to the blastocyst stage. This aspect of the invention therefore includes a cell, a cell line, or an embryo prepared according to a method as described above, and an animal prepared by allowing such an embryo to develop to term in the final recipient animal.

(b) Application of invention in methods of *in vitro* production of pigs

In vitro production can include any or all of the following three stages: (1) *in vitro* maturation (IVM) of the oocyte; (2) *in vitro* fertilization (IVF) of the oocyte; and/or (3) *in vitro* culture (IVC) of the fertilized oocyte, generally to the blastocyst stage. Generally, the oocyte to be fertilized will be subjected to *in vitro* maturation step prior to being brought into proximity with the sperm cell. *In vitro* production of an animal embryo will typically involve *in vitro* fertilization of animal oocytes and can be achieved by any suitable method depending upon the animal species. Examples of such methods include, but are not limited to those of Trounson *et al Theriogenology* 41 57-66 (1994); Thompson, J. G. *Reprod. Fertil. Dev.* 9 341-354 (1997); Wilmut *et al* in *Genetics of the Sheep*, pages 395-412 ed.s Piper, L. and Ruvinsky, A., CAB International, Oxford, UK (1997); Summers *et al Biol. Reprod.* 53 431-437 (1995); Weston, A. M. and Wolf, D. P. *Mol. Reprod. Dev.* 44 88-92 (1996); Liu *et al Mol. Reprod. Dev.* 45 157-162 (1996); Li *et al Theriogenology* 47 1103-1113 (1997); Trounson, A. and Gardner, D. K. ed.s *Handbook of in vitro Fertilisation*, CRC Press Inc. Salem, USA (1993)). This aspect of the invention may also involve *in vitro* culture of the resultant animal embryos. It will also be readily appreciated that a method according to this aspect of the invention may also include further screening steps for the viability of the embryo and its suitability for transfer to the final female recipient. Additionally, the method also extends to a cell or a cell line prepared from a porcine embryo according to a method of the invention.

(c) Application of method to transplantation therapies

A pig prepared by a method in accordance with any aspect of the present invention may be used as a source of tissue for transplantation therapy. Similarly, a pig embryo prepared in this manner or a cell line developed therefrom may also be used in cell-transplantation therapy. Accordingly, there is provided in a further aspect of the invention a method of therapy comprising the administration of porcine cells to a patient, wherein the cells have been prepared from an embryo or animal prepared by a method as described above. This aspect of the invention extends to the use of such cells in medicine, e.g. cell-transplantation therapy, and also to the use of cells derived from such embryos in the preparation of a cell or tissue graft for transplantation. The cells may be organised into tissues, for example, heart, lung, liver, kidney, pancreas, corneas, nervous (e.g. brain, central nervous system, spinal cord), skin, or the cells may be blood cells (e.g. haemocytes, i.e. red blood cells, leucocytes) or haematopoietic stem cells or other stem cells (e.g. bone marrow). A method of the present invention will therefore also find utility in the preparation of xenografts. These methods might include *in vitro* differentiation of embryonic cells for therapeutic transplantation into a patient, including situations where the cells are genetically modified to correct a medical defect. Such applications might include treatment of diseases such as diabetes, Parkinson's disease, motor neurone disease, multiple sclerosis, AIDS etc, or disease conditions characterised by a loss of function in the cells or an organ of an affected individual.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

EXAMPLES

The invention will now be further described by way of reference to the following Examples and Figures which are provided for the purposes of illustration only and are not to be construed as being limiting on the invention. Reference is made to a number of Figures in which:

FIGURE 1 shows results of initial experiment to monitor the response of gilts to superovulatory stimuli in two different regimes. In regime 1 (□),

Pregnant Mares Serum Gonadotropin (PMSG) / Prostaglandin injection was given at 5.00pm local standard time in the UK, followed by an injection of human chorionic gonadotropin (hCG) after 88 hours at 9.00am local standard time in the UK. In regime 2 (■), Pregnant Mares Serum Gonadotropin (PMSG) / Prostaglandin injection was given at 7.00pm local standard time in the UK, followed by an injection of human chorionic gonadotropin (hCG) after 88 hours at 11.00am local standard time in the UK. For both regimes, the PMSG/Prostaglandin injection was given 88 hours prior to the hCG injection.

FIGURE 2 shows results from expanded experiment to monitor the response of gilts to superovulatory stimuli in two different regimes as in Figure 1.

FIGURE 3 shows parthenogenetic development to blastocyst of ovulated porcine oocytes following electrical activation with reference to the time of ovulation.

FIGURE 4 shows parthenogenetic development to blastocyst of porcine oocytes matured *in vitro* following electrical activation with reference to the period of maturation *in vitro*.

Example 1: Developmental competence of oocytes collected after ovulation *in vivo*:

Synchronization of oestrus and superovulation

Cycling gilts were fed 5mg of altrenogest daily for 18 days, which was followed by daily heat detection to establish a reference heat. Between 13-16 days after reference heat gilts were injected with 1500 IU of pregnant mares serum gonadotropin (PMSG) and 2ml (175µg) of cloprostinol (synthetic prostaglandin) at an appropriate time. Eighty-eight hours later this was followed with an injection of 500 IU of human chorionic gonadotropin (hCG). To delay the time of ovulation the timing of PMSG/hCG injections was shifted from the standard 5pm (PMSG) and 9am (hCG) by 2 hours to 7pm (PMSG) and 11am (hCG).

Detection of ovulation

Ovulation was detected by transcutaneous ultrasonography using a real-time B-mode ultrasound machine with a 5mhz convex linear probe. Scanning was carried out from 38hours post hCG injection every 2 hours until 49hours post-hCG injection. Ovulation was deemed to have occurred when the majority of the follicles had disappeared.

Collection of oocytes

The oocytes were collected by performing a mid-line laparotomy on gilts under general anaesthesia. The reproductive tract was exposed, and following canulation the oviduct was flushed with collection medium (Calcium-free Hepes buffered M2 medium; Hogan *et al* in "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbour Laboratories, New York (1986)) prewarmed to 39°C. The collection medium may also be Hepes buffered M2 containing calcium or NCSU medium (Wang *et al J. Reprod. Fertil.* 111 101-108 (1997)), either with or without calcium.

Activation and culture of oocytes

Oocytes were activated electrically in 0.3M Mannitol, 100µM MgCl₂, 50µM CaCl₂, using 3 pulses of 80µS duration at 1.25 kV/cm. This was followed by 3 washes in Hepes buffered MR2 medium and then culture for 5 hours in NCSU medium containing 1x BME Amino Acids (Sigma), 1x MEM non-essential amino acids (Sigma), 4mg/ml Bovine Serum Albumen, 100µg/ml cysteine and 7.5µg/ml cytochalasin B at 39°C in a 5% O₂, 5% CO₂, 90% N₂ environment (Abeydeera *et al., Biol. Reprod.* 58 213-218 (1998)). Activated oocytes were then washed free of and cultured in the same culture medium lacking cytochalasin B, in 20µl microdrops under paraffin oil and the same gas atmosphere and temperature described above for 7 days.

Results

The above methods were applied to mature gilts. In an initial set of experiments, 14 to 16 animals were allocated to each of two regimes wherein ovulation was induced by injection of PMSG and hCG. The regimes differed by a shift of 2 hours in the commencement of the protocol. This 2 hour shift resulted in a prolonging of the modal ovulation time by approximately 3 hours (see Figures 1 and 2). The development of parthenogenetically activated oocytes to the blastocyst stage was followed after 7 days of culture. Interestingly, the developmental competence of the oocytes correlated with the shift in modal ovulation time. A striking positive correlation was observed between the percentage of oocytes reaching the blastocyst stage (an endpoint of preattachment development) and the time with which those oocytes were ovulated (see Figure 3). This trend demonstrates that oocytes which are ovulated later exhibit increased competence for embryonic development within a defined time period or window. The method described to prolong the onset of ovulation, which likely resulted from an interaction between the injected gonadotropins and diurnal variations in female responsiveness to these hormones, provides a means to improve the yield of developmentally competent oocytes for both natural and assisted reproductive strategies.

Example 2: Developmental competence of oocytes matured *in vitro*

Collection of oocytes

Ovaries were collected from a slaughterhouse into a thermal box containing gel pads pre-warmed to approximately 30°C. If the temperature of ovaries and pads is greater than 30°C then pads are removed. During collection and transport, temperature of ovaries should be within 25-30°C. On arrival at the laboratory, ovaries were washed 2X with PBS and maintained in a beaker in a water bath at 25°C during follicular aspiration. Cumulus oocyte complexes (COCs) were aspirated from 3-8mm follicles using a 18 gauge needle attached to a 10ml syringe and follicles were pierced from an interior surface. Aspirate is collected in sterile plastic universal vials. Needles should be temporarily removed when dispensing contents of syringe into vials. Aspirations should be completed within an hour of ovary arrival.

Collected COCs were washed 3X with TL-Hepes-PVA by draining all but one third of aspirate, and adding 5-10mls of TL-Hepes-PVA (prewarmed to 39°C). After allowing 1-2 minutes for COCs to settle during each wash, the process is repeated.

- 5 Washed COCs were then sorted in sterile 60 mm dishes. Only COCs that are completely enclosed by non-expanded cumulus were selected. Dark, denuded oocytes were not selected. After selection COCs were washed through 3 further changes of TL-Hepes-PVA, followed by passage through 3x 500µl volumes of Maturation medium lacking hormones in 4 well Nunc™ plate. Maturation medium
10 consisted of 0.22µm filter sterile, bicarbonate buffered North Carolina State University (NCSU23) medium (Wang *et al* (*J. Reprod. Fertil.* 111 101-108 (1997)) supplemented with 1x Basic Minimal Essential (BME; Sigma B-6766) and 1x Minimal Essential (MEM; Sigma M-7145) amino acids, and 10% pig follicular fluid.

15 ***In vitro* maturation**

- For maturation COCs were cultured for 22 hours in Maturation medium containing the hormones human Chorionic Gonadotropin (hCG) and Equine Chorionic Gonadotropin (eCG) to a final concentration of 10 IU/ml, respectively, followed by another 16 to 26 hours in maturation medium without hormones. All maturation was
20 at 39°C in a 5% CO₂ in air environment.

Activation

- Prior to parthenogenetic activation oocytes were denuded of expanded cumulus by pipetting in Hepes buffered NCSU23 containing 600 IU/ml hyaluronidase for 2 to 3
25 minutes at room temperature in groups of 50. Denuded oocytes were then washed in Calcium-free hbNCSU23 two times followed by passage through 2 volumes of activation medium (0.3M Mannitol, 50µM CaCl₂, 100µM MgCl₂). In groups of 25 washed oocytes were then loaded into an electrical activation chamber containing 200µl of Mannitol over electrodes. Oocytes were aligned in single row, and were
30 then subject to a pulse of 5 seconds x 5V AC to further align them, followed by 3x80 µsec pulses of DC current at a field strength ranging from 1 to 1.5 KV/cm (preferably 1 KV/cm).

Electrically activated oocytes were next transferred into NCSU23 containing 7.5µg/ml Cytochalasin B (or equivalent inhibitor of cytokinesis) for 6-8 hours at 39°C, 5% CO₂. After this treatment which is intended to suppress the emission of a second polar body, thus resulting in the creation of eggs which are karyotypically diploid, eggs were washed through 3 volumes of NCSU23 before finally being cultured in the same medium for up to 7 days at 39°C, 5% CO₂ in air.

Preparation of Pig Follicular Fluid for Archiving

After permitting follicular aspirate to settle in universal tube, top fluid was drawn off and centrifuged at room temperature for 30 minutes at 3000rpm (1500g). Supernatant was frozen as 1ml aliquots at -20°C. Frozen aliquots can be used to prepare maturation medium in future experiments.

Studies on activation conditions for *in vitro* matured oocytes

Although 100% of *in vitro* matured pig oocytes can be activated parthenogenetically, the rate of blastocyst formation is still low. Experiments were carried out to investigate the best conditions for pig oocyte activation in order to improve its efficiency. In all experiments, pig oocyte maturation was undertaken according to a method described by Wang *et al* (*J. Reprod. Fertil.* 111 101-108 (1997)).

The timing of oocyte maturation in the *in vitro* maturation (IVM) system was studied as follows. Oocytes were fixed in acetic acid:methanol and stained by 1% orcein at 0, 22, 36, 37, 38, 39, 40, 41, 42 and 43 hours of maturation respectively to assess nuclear maturation. 82% of 50 oocytes arrested at GV stage at 0h, 68% of 44 oocytes at MI stage at 22 hours, and 75% of 36 oocytes reached MII stage at 36 hours, after 37 hours and 43 hours more than 90% of oocytes were MII stage, confirming the efficiency of IVM.

Activation and culture of oocytes

The effects of different culture media and activation conditions were also investigated. In all experiments, activated oocytes were cultured in 7.5 µg/ml

cytochalasin B for 6 hours, then transferred into NCSU23 medium + 0.4% BSA and cultured for 6 or 7 days, at 39°C, in 5%CO₂ in air assess development to blastocyst stage.

5 (i) The effect of oocyte age and field strength were examined as follows. Oocytes were activated at 36, 40, 44 and 48 hours, with 1 pulse of 1.0, 1.25 and 1.5kV/cm DC for 80µs, respectively 0.3M Mannitol, 0.1mM Mg²⁺, 0.05mM Ca²⁺ and were then cultured for 6 days. The results show that blastocyst rate increased from 36 to 44 hours of maturation in all the treatments (range from 14.5 to 41.1%), of which, 10 the best result was 41.1% using 1 pulse of 1.5kV/cm at 44 hours of maturation (see Figure 4). Interestingly, at 48 hours the developmental competence of oocytes activated at virtually all electrical field strengths dropped. This demonstrates the existence of the limits of a window of developmental competence comparable to that seen in *in vivo* derived oocytes (see Figure 3).

15

(ii) In this experiment pig oocytes matured for 44 hours were activated in the same medium as that in experiment (i) by combinations of 1, 3, 5 (x 80µs) pulse(s) of 1.0kV/cm, 1.25 kV/cm and 1.5kV/cm DC, respectively, following 0.25kV/cm AC for 5 seconds. Blastocyst rate was significantly higher (54.3%) in the oocytes 20 activated by 3 pulses of 1.0 kV/cm than those using other treatments (below 42.2%).

(iii) In this experiment, pig oocytes matured for 44 hours were activated in:

- 0.3M Mannitol alone (1),
- 0.3M Mannitol and 0.1mM Mg²⁺ (2),
- 25 0.3M Mannitol, 0.1mM Mg²⁺ and 0.05mM Ca²⁺ (3); and
- 0.3M Mannitol, 0.1mM Mg²⁺ and 0.1mM Ca²⁺ (4).

Activation was carried out using 3 pulses (x80µs) of 1.0k V/cm DC following 0.25kV/cm AC for 5 seconds, followed by culture for 7 days. The medium (3) gave 30 the best rate of blastocyst (44%) compared to the other media although the lowest rate was only 14.4% in the medium (2).

(iv) In this experiment, pig oocytes matured for 44 hours were activated in the medium (3), Sorbitol + 0.1mM Mg^{2+} + 0.05mM Ca^{2+} and Zimmermann medium, respectively, by 3 pulses ($\times 80\mu s$) of 1.25kV/cm following 0.25kV/cm AC for 5 seconds, the activated oocytes were then cultured for 7 days. No differences in blastocyst rate and mean cell numbers per blastocyst were observed among the three media (36.1%, N=374, 45.39 ± 15.88 for medium 3, 35.4%, N=381, 51.70 ± 20.72 for Sorbitol; and 40.62%, N=357, 46.12 ± 21.14 for Zimmermann medium).

CLAIMS

1. A method of producing a porcine oocyte optimised for developmental competence, the method comprising maturing a harvested oocyte *in vitro* for between about 36 to about 48 hours.
5
2. A method as claimed in claim 1, in which the *in vitro* maturation is for between about 42 to about 46 hours
3. A method as claimed in claim 1 or claim 2, in which the *in vitro* maturation
10 is for between about 43 to about 45 hours.
4. A method of producing a porcine oocyte optimised for developmental competence, the method comprising harvesting an oocyte from a pig from about 42 to about 50 hours post-injection with a gonadotropin hormone, wherein the oocyte
15 was ovulated from about 42 to about 50 hours post-gonadotropin injection.
5. A method as claimed in claim 4, in which the oocyte is harvested from the pig from about 44 to about 48 hours post-injection with a gonadotropin hormone, wherein the oocyte was ovulated from about 44 to about 48 hours post-gonadotropin
20 injection.
6. A method as claimed in claim 4 or claim 5, in which the oocyte is harvested from the pig from about 45 to about 47 hours post-injection with a gonadotropin hormone, wherein the oocyte was ovulated from about 45 to about 47 hours post-gonadotropin injection.
25
7. A method as claimed in any one of claims 4 to 6, in which ovulation of the oocyte is detected using transcutaneous ultrasonography.
8. A method of harvesting an oocyte from a porcine mammal in response to the
30 administration of a gonadotropin hormone, the method comprising administration of

the gonadotropin hormone towards the end of the diurnal rhythm of the porcine mammal, and subsequently harvesting the ovulated oocyte.

- 5 9. A method of enhancing the developmental competence of a harvested porcine oocyte, the method comprising administration of a gonadotropin hormone to a porcine mammal towards the end of the diurnal rhythm of the animal prior to harvesting the oocyte such that ovulation is delayed.
- 10 10. A method of nuclear transfer, the method comprising the introduction of a nucleus from a donor cell into a porcine oocyte produced by a method as defined in any one of claims 1 to 7.
- 15 11. A method of *in vitro* production of a porcine embryo, the method comprising the step of *in vitro* fertilization (IVF) of an oocyte produced by a method as defined in any one of claims 1 to 7.
- 20 12. A composition of porcine oocytes selected for optimal developmental competence, wherein the oocytes substantially comprise oocytes matured *in vitro* for between about 36 to about 48 hours.
- 25 13. A composition as claimed in claim 12, in which the oocytes substantially comprise oocytes matured *in vitro* for between about 42 to about 46 hours.
14. A composition as claimed in claim 12 or claim 13, in which the oocytes substantially comprise oocytes matured *in vitro* between about 43 to about 45 hours.
- 30 15. A composition as claimed in claim 12, in which at least about 50% of the oocytes were matured *in vitro* for about 36 to about 48 hours.
16. A composition of porcine oocytes selected for optimal developmental competence, wherein the oocytes substantially comprise oocytes harvested from at

least one pig from about 42 to about 50 hours post-injection with a gonadotropin hormone, wherein the oocyte was ovulated from about 42 to 50 hours post-gonadotropin injection.

- 5 17. A composition as claimed in claim 16, in which the oocytes substantially comprise oocytes harvested from the at least one pig from about 44 to about 48 hours post-injection with a gonadotropin hormone, wherein the oocytes were ovulated from about 44 to about 48 hours post-gonadotropin injection.
- 10 18. A composition as claimed in claim 16 or claim 17, in which the oocytes substantially comprise oocytes harvested from the at least one pig from about 45 to about 47 hours post-injection with a gonadotropin hormone, wherein the oocytes were ovulated from about 45 to about 47 hours post-gonadotropin injection.
- 15 19. A composition as claimed in claim 16, in which at least about 50% of the oocytes were harvested from the at least one pig from about 42 to about 50 hours, post-injection with a gonadotropin hormone, wherein the oocytes were ovulated from about 42 to about 50 hours post-gonadotropin injection.
- 20 20. A composition as claimed in any one of claims 16 to 19, in which ovulation of the oocyte is detected using transcutaneous ultrasonography.
- 21 A method of producing a porcine oocyte optimised for developmental competence, the method comprising:
- 25 (i) harvesting a cumulus oocyte complex (COC) from a follicle of a pig ovary; and
- (ii) maturing said COC in a physiologically acceptable culture medium comprising a gonadotropin hormone for between about 36 hours to about 48 hours.

22. A method of producing a porcine oocyte optimised for developmental competence, the method comprising:

- (i) administering progesterone or an analogue thereof to a pig for 18 days to establish a reference heat in the animal;
- 5 (ii) thirteen to sixteen days subsequently administering to the animal a first injection of Pregnant Mares Serum Gonadotropin (PMSG);
- (iii) after a time period of about 88 hours administering to the animal a second injection of human chorionic gonadotropin (hCG); and
- 10 (iv) harvesting an oocyte from a pig from about 42 hours to about 50 hours post-injection with hCG, wherein the oocyte was ovulated from about 42 hours to 50 hours post-hCG injection as determined by transcutaneous ultrasonography.

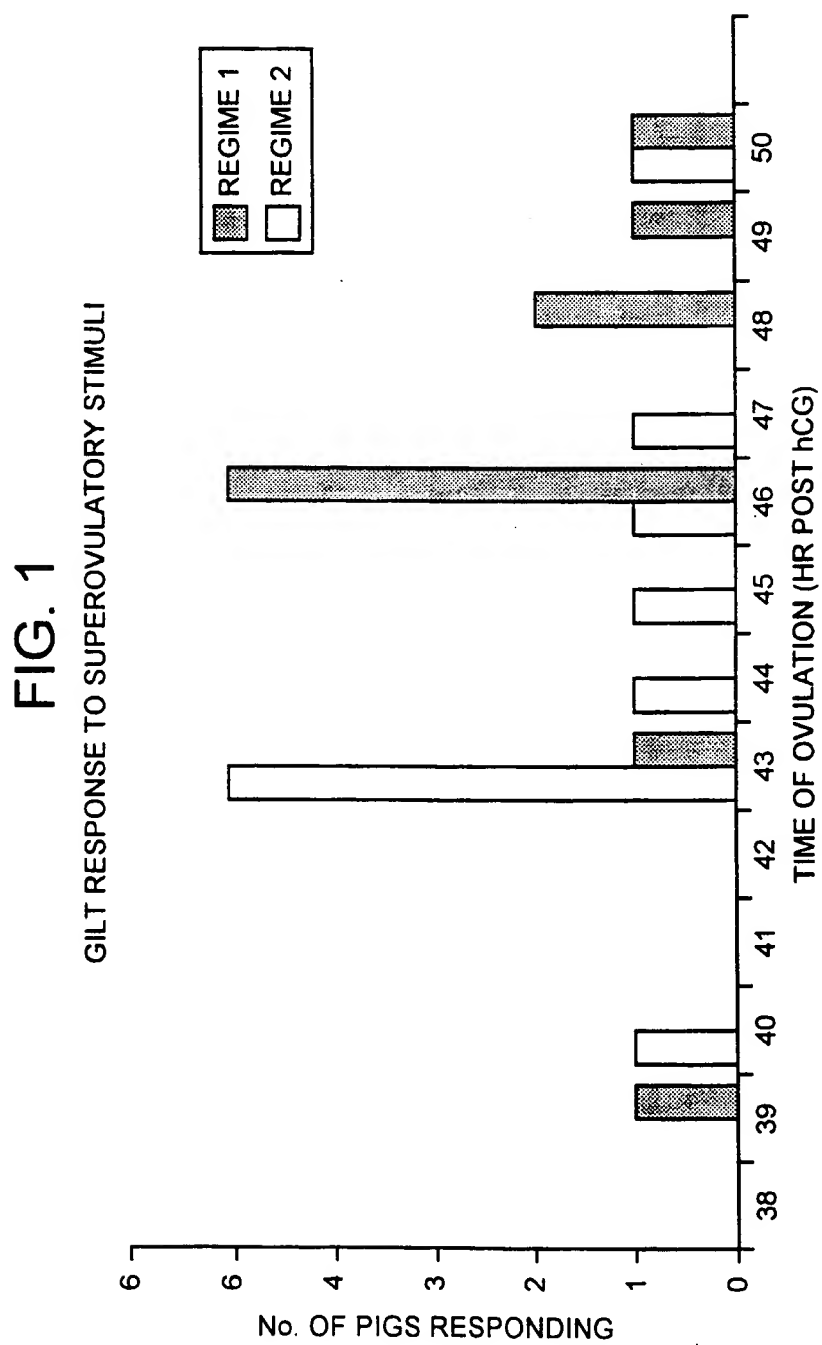
23. A method of producing a porcine oocyte optimised for developmental competence, the method comprising:

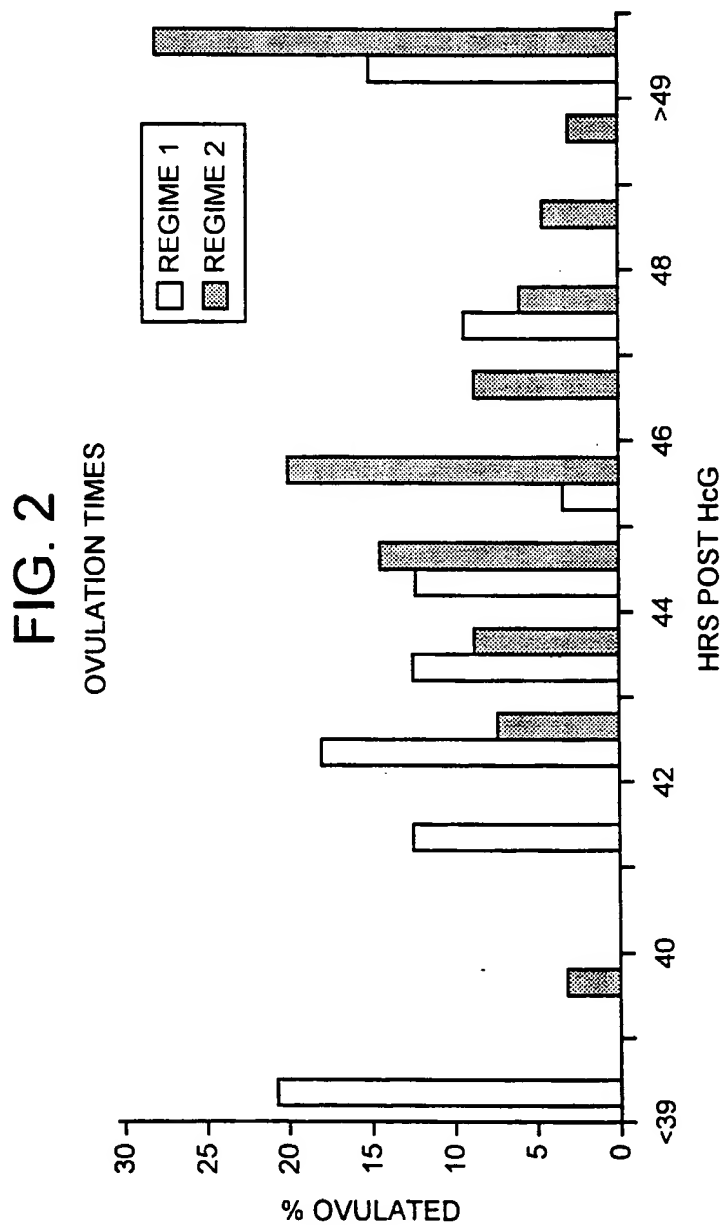
- (i) administering to the animal progesterone or an analogue thereof for four or five days at days 11 to 15 of the estrous cycle;
- (ii) subsequently administering to the animal PMSG one day later and hCG four days later to cause ovulation of an oocyte; and
- 20 (iii) harvesting an oocyte from a pig from about 42 to about 50 hours post-injection with hCG, wherein the oocyte was ovulated from about 42 to 50 hours post-hCG injection as determined by transcutaneous ultrasonography.

25 24. The use of separate first and second administrations of gonadotropin hormone to harvest an oocyte from a porcine mammal in which the first administration of gonadotropin hormone is towards the end of the diurnal rhythm of the animal.

25. The use of a gonadotropin hormone in the manufacture of an agent for the harvesting of an oocyte from a porcine mammal whereby the gonadotropin hormone is administered towards the end of the diurnal rhythm of the animal.
- 5 26. A kit comprising a gonadotropin hormone wherein the kit contains instructions for administration of the gonadotropin hormone to a porcine mammal for harvesting an oocyte from the animal in which administration of the hormone is towards the end of the diurnal rhythm of the animal.
- 10 27. A kit comprising a first gonadotropin hormone and a second gonadotropin hormone wherein the kit contains instructions for administration of the gonadotropin hormones to a porcine mammal for harvesting an oocyte from the animal in which administration of the first hormone is towards the end of the diurnal rhythm of the animal and administration of the second hormone is separated from the first
- 15 administration by about three days.

1 / 4





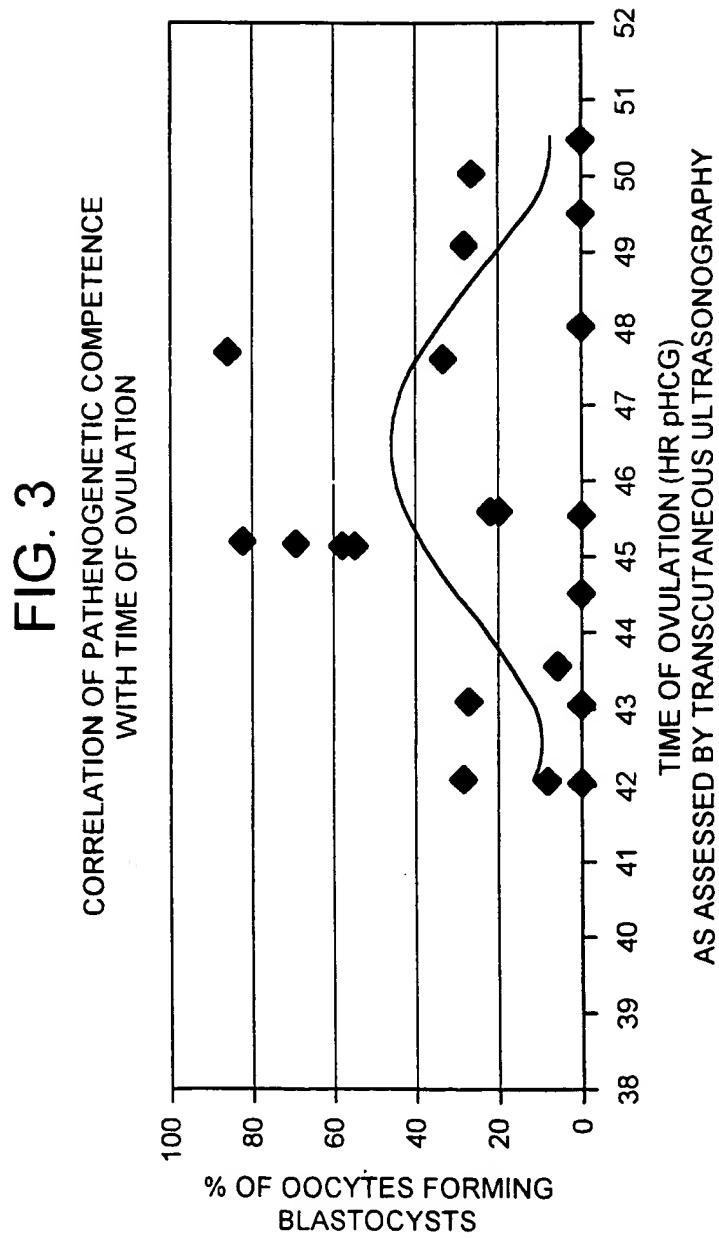
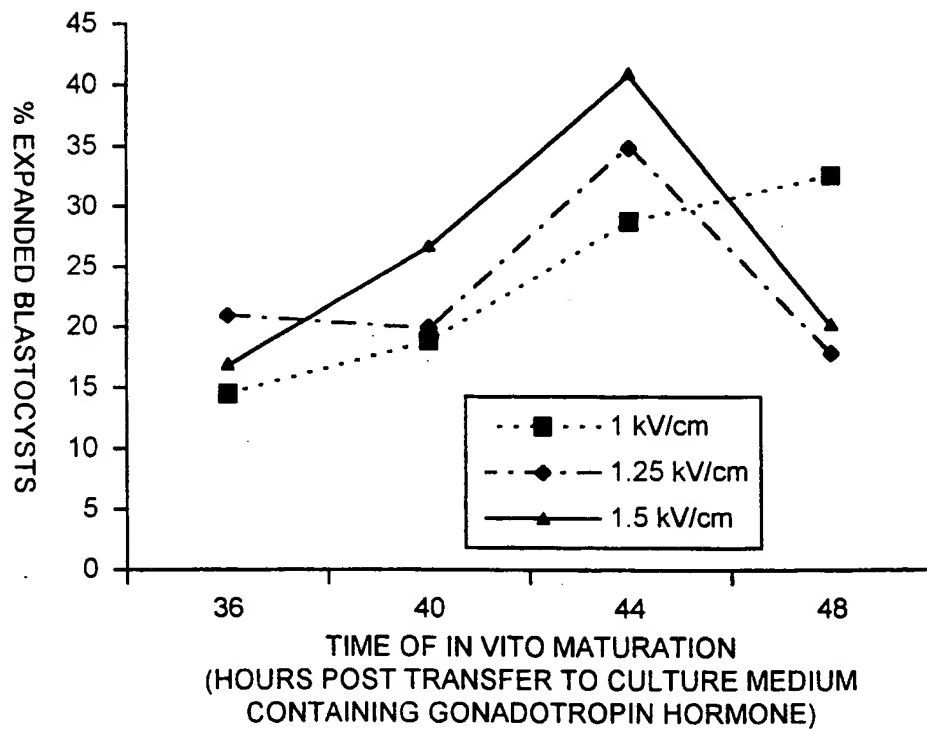


FIG. 4

PARTHENOGENETIC DEVELOPMENT OF IVM PORCINE OOCYTES
FOLLOWING SINGLE PULSE ELECTRICAL ACTIVATION



INTERNATIONAL SEARCH REPORT

Int: ional Application No

PCT/GB 99/03384

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C12N15/89 A61K35/54 A61K38/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRUPEN C G ET AL: "ROLE OF EPIDERMAL GROWTH FACTOR AND INSULIN-LIKE GROWTH FACTOR-I ON PORCINE OOCYTE MATURATION AND EMBRYONIC DEVELOPMENT IN VITRO" REPRODUCTION, FERTILITY AND DEVELOPMENT, vol. 9, no. 6, page 571-575 XP000857263 ISSN: 1031-3613 page 572, left-hand column, paragraph 2	1-3, 12-15
X	NAGASHIMA H ET AL., : "Nuclear transfer of porcine embryos using cryopreserved delipated blastomeres as donor nuclei" MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 48, no. 3, November 1997 (1997-11), pages 339-343, XP000884362 abstract page 340 -page 341	1,4,10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

1 March 2000

Date of mailing of the international search report

09/03/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Mateo Rosell, A.M.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03384

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SOEDE N M ET AL., : "Ultrasonography of pig ovaries: Benefits in research and on farms." REPRODUCTION IN DOMESTIC ANIMALS, vol. 29 (5), 1994, page 366-370 XP000884409 the whole document</p>	7,22
A	<p>WO 95 28412 A (GUSTAFSSON KENTH T ; INST OF CHILD HEALTH (GB); BAETSCHER MANFRED W) 26 October 1995 (1995-10-26) abstract page 3, last paragraph -page 4, line 2 page 5, paragraph 2; example 5 page 31, paragraph 2 -page 32, last paragraph</p>	1,4,10, 11,22,23
A	<p>MACHATY Z ET AL: "PARTHENOGENETIC ACTIVATION OF PORCINE OOCYTES WITH GUANOSINE-5 -O- (3 -THIOTRIPHOSPHATE)" BIOLOGY OF REPRODUCTION,US,ORLANDO, FL, vol. 52, no. 4, 1995, page 753-758 XP000775994 ISSN: 0006-3363 page 753, last paragraph -page 754, paragraph 3</p>	1-3,10
A	<p>PRATHER RS ET AL., : "Artificial activation of porcine oocytes matured in vitro" MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 28, no. 4, April 1991 (1991-04), pages 405-409, XP000884363 abstract page 406 -page 407</p>	1,10
A	<p>GB 1 398 022 A (AMERICAN OPTICAL CORP) 18 June 1975 (1975-06-18) column 1, line 13-40</p>	7
A	<p>WO 97 07668 A (CAMPBELL KEITH HENRY STOCKMAN ; ROSLIN INST EDINBURGH (GB); WILMUT) 6 March 1997 (1997-03-06) page 5, line 1-24 page 7, line 4-25 page 10, line 11 -page 11, line 5 page 12, line 10-20 page 21-28</p>	1,10
P,X	<p>WO 99 46982 A (NOTTLE MARK BRENTON ; BRESAGEN LTD (AU); VERMA PAUL JOHN (AU)) 23 September 1999 (1999-09-23) page 3, line 13-23; examples 1-5</p>	1,10,11
	-/--	

INTERNATIONAL SEARCH REPORT

Int. Jonal Application No
PCT/GB 99/03384

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>WO 99 01164 A (UNIV MASSACHUSETTS A PUBLIC IN) 14 January 1999 (1999-01-14) page 8, line 10 -page 12, line 23 page 31-43</p> <p>-----</p>	1,10

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/03384

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9528412 A	26-10-1995	AU 1850599 A AU 2233295 A CA 2187802 A EP 0755402 A JP 10504442 T	29-04-1999 10-11-1995 26-10-1995 29-01-1997 06-05-1998
GB 1398022 A	18-06-1975	CA 995762 A CH 543876 A DE 2244762 A FR 2159076 A JP 48050581 A NL 7213632 A	24-08-1976 28-12-1973 03-05-1973 15-06-1973 17-07-1973 02-05-1973
WO 9707668 A	06-03-1997	AU 6830996 A CA 2229657 A CN 1202085 A CZ 9800604 A EP 0847237 A GB 2318792 A GB 2340493 A HU 9802485 A NO 980846 A PL 325336 A	19-03-1997 06-03-1997 16-12-1998 15-07-1998 17-06-1998 06-05-1998 23-02-2000 01-02-1999 29-04-1998 20-07-1998
WO 9946982 A	23-09-1999	AU 2913599 A	11-10-1999
WO 9901164 A	14-01-1999	AU 8374598 A	25-01-1999